Biochemical Investigation of Cell Motile Activity in Rheumatoid Synovial Fluid

KIMBHIKO TAKEUCHI, HIDEOMI WATANABE, and KENJI TAKAGISHI

ABSTRACT. Objective. We have suggested that autocrine motility-like factor is expressed in rheumatoid synovial fluid (SF). We examined the biochemical features of the motile activity.

Methods. We investigated chemotactic activities of SF from patients with osteoarthritis and joint trauma as well as rheumatoid arthritis (RA) using a unique protein-free culture fibrovascular system. We then investigated biochemical features and the signal transduction pathway of the motile activity expressed in RA.

Results. We found chemokinetic activities in SF from all patients. However, inability to block binding of the monoclonal antibody to the receptor for autocrine motility factor (AMF) was observed only in rheumatoid SF on immunoblotting. Biochemical investigation indicated motile activity to be heat labile, trypsin sensitive, and eluted at high salt concentration from an anion exchange column. Stimulated motility of rheumatoid SF was inhibited by the permisooxin, staurosporine (C kinase inhibitor), and genistein (tyrosine kinase inhibitor), but not by A kinase inhibitor, H-8.

Conclusion. The cell motility activity expressed in rheumatoid SF appears to be AMF, and the cytokine may be essential for communication among leukocytes in rheumatic disease. (J Rheumatol 1998;25:615-16)

Key Indexing Terms:
RHEUMATOID ARTHRITIS SYNOCVIAL FLUID AUTOCORINE MOTILITY FACTOR CELL MOTILITY

Rheumatoid arthritis (RA) is a chronic, persistent granuloma forming inflammatory disease, and several cell types are present in RA joint fluid that cause joint destruction.1 Cytokines controlling cell motile activity are associated with the presence of inflammation related cells.2 Preinflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), IL-6, and IL-8 are synthesized and secreted by activated macrophages into the synovium.3,4 TNF-α and IL-8 are directly essential for polymorphonuclear leukocyte recruitment (PMN) and lymphocyte recruitment.5 Although less important in this regard, IL-1 and TNF-α are strong inducers of IL-8, and the former would thus appear to contribute to leukocyte recruitment indirectly. Granulocyte macrophage colony stimulating factor (GMCSF) is present in rheumatoid synovial fluid (SF) and stimulates IL-1 production.6 The relative contribution of such a chemotaxis related factor in the pathogenesis of RA is complex, as suggested by Brennan, et al., and has yet to be fully determined.

Autocrine motility factor (AMF) has been identified biochemically as a specific cytokine that stimulates random and directed motion of self-producing cells.7 The motile activity has been purified from the culture media of various tumor cells8–10, and its molecular homology with neurotensin/phosphohexose isomerase has been revealed.11 A 78 kDa glycoprotein (gp78) has been shown to function as a receptor for AMF and to have the structural features of an integral membrane protein.12 The expression of gp78 is associated with the invasive properties of various malignant tumors14–15. The AMF receptor is expressed in murine fibroblasts and plays a role in the motility of the fibroblast16. The AMF–gp78 signaling pathway would thus not be unique to biological processes in tumor cells. The expression of AMF-like motile activity was observed in rheumatoid SF using a unique protein-free culture cell line.17 As well, we have established a procedure for the purification of AMF13. In the present study, we examined motile activities in SF of patients with osteoarthritis (OA), joint trauma, and RA, and investigated biochemical features of the motile activity expressed in RA. The presence of this activity was found to be consistent with that of AMF reported previously.

MATERIALS AND METHODS

Patients. SF was obtained from 4 subjects with RA, all meeting the American Rheumatism Association criteria for definite or classical RA. 4 subjects with OA, and 4 with joint trauma. No patient had a malignant tumor or neoplastic intravascular malformation during the previous 6 months. Fluid and serum samples were obtained as described.17 Briefly, each sample was aspirated aspirally from the suprapatellar pouch and intrarti-
RESULTS

Protein concentrations of SF. Mean values for protein concentration in SF and standard deviation were 48.7 ± 17.9, 29.7 ± 5.3, and 27.5 ± 7.9 mg/ml in RA, OA, and joint trauma, respectively. The protein concentration of rheumatoid SF was significantly higher than that of OA or joint trauma samples.

Phagocytic response of Gc-4 PF cells to SF. As shown in Figure 1, the average areas of the particle-clear zone formed by Gc-4 PF cells in the absence or presence of rheumatoid SF at protein concentrations of 0, 100, 250, and 500 µg/ml were 10.1 ± 0.9, 14.1 ± 0.7, 16.8 ± 0.9, and 20.5 ± 1.7 µm², respectively. Control data indicated the basal phagocytic response of Gc-4 PF cells without SF. This dose-dependent chemotactic response was commonly observed in all RA samples (data not shown). Phagocytic motility was significantly stimulated by OA and joint trauma SF (Table 1), indicating cell motility stimulation activity in not only RA but in OA and joint trauma SF as well. Next, we examined the motile activities of serum from patients with RA or OA compared with those of SF. Of interest, serum from patients with OA did not stimulate cell motility, while serum as well as SF from patients with RA stimulated cell motility almost equally (Table 2). Binding of cell motility activity in rheumatoid SF to gp78. In immunoblot analysis, the incubation of Gc-4 PF cell extracts on nitrocellulose filters with anti-gp78 Mab resulted in clear labeling of gp78 (Figure 2A), while incubation of the filter with RA SF before adding the anti-gp78 Mab decreased the labeling of gp78 (Figure 2B). Thus, there is a competitive inhibitor of anti-gp78 Mab against gp78 or gp78 itself in RA SF. In vitro analysis was performed to determine whether rheumatoid SF contains molecules recognized by anti-gp78 Mab. Figure 3 shows the incubation of the filter with anti-gp78 Mab did not change the intensity of that anti-gp78 Mab, indicating gp78 itself or gp78-like molecules recognized by the antibody were not present, but a factor binding to gp78 may exist in rheumatoid SF. SF from OA (Figure 2C) or patients with joint trauma (data not shown) did not inhibit the binding of anti-gp78 Mab to the AMF receptor, suggesting that molecular binding to gp78 may be specific for RA SF.

Biochemical features of cell motility activity in RA SF. The chemotactic activity of rheumatoid SF decreased in a time-dependent manner after incubation with trypsin, and was heat-labile (Figure 4). We have reported the results of fibroblast cell culture medium treated with anti-exchange chromatography and buffer as in this study. AFM was recovered as an unbound fraction and a fraction eluted at high salt concentration (500 mM) and protenase (10 mg/ml) prepared in dimethyl sulfoxide (DMSO) was dialyzed with culture medium and used as control. DMSO at a similar concentration was used in the control.

Table 1. Phagocytic response of Gc-4 PF cells to synovial fluid (500 µg/ml) from patients with RA, OA, or joint trauma. All data are expressed as mean ± standard error.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Cell Motility (µm²/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>5.3 ± 0.3</td>
</tr>
<tr>
<td>RA 3</td>
<td>11.4 ± 1.0</td>
</tr>
<tr>
<td>1</td>
<td>13.3 ± 2.7</td>
</tr>
<tr>
<td>2</td>
<td>13.2 ± 2.1</td>
</tr>
<tr>
<td>OA</td>
<td>15.0 ± 0.9</td>
</tr>
<tr>
<td>Joint trauma</td>
<td>25.9 ± 4.9</td>
</tr>
<tr>
<td>1</td>
<td>16.3 ± 2.7</td>
</tr>
<tr>
<td>2</td>
<td>16.0 ± 2.2</td>
</tr>
</tbody>
</table>

*P<0.001 vs Control

Table 2. Phagocytic response of Gc-4 PF cell to synovial fluid or serum from patients with RA or OA. All data are expressed as mean ± standard error.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Cell Motility (µm²/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.3 ± 0.3</td>
</tr>
<tr>
<td>RA 3</td>
<td>11.2 ± 1.1</td>
</tr>
<tr>
<td>Serum</td>
<td>9.9 ± 0.8</td>
</tr>
<tr>
<td>OA 1</td>
<td>12.9 ± 3.1</td>
</tr>
<tr>
<td>Serum</td>
<td>6.7 ± 0.4</td>
</tr>
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*P<0.001 vs Control

Involvement of guanine nucleotide binding (G) protein and specific kinase reactions in cell motility stimulated by rheumatoid SF. Pertussis toxin causes a profound, rapid decrease in motility stimulated by AMF. The precipitination of Gc-4 PF cells with 500 ng/ml pertussis toxin caused a significant reduction in cell motility stimulated by rheumatoid SF (Figure 5). With pre-incubation of cells with 10 µM staurosporin, a C kinase inhibitor, or 10 µg/ml genistein, a tyrosine kinase inhibitor, had the same result. Reagent dose was basically the same as that which inhibited the AMF stimulated motility. In contrast, no significant inhibition of stimulated motility was observed upon the addition of 10 µM H-8.

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Figure 2. Immunoblot analysis. Gc-4 PF cell extracts equivalent to 2 x 10^6 cells were electrophoresed onto polyacrylamide gels and transferred to nitrocellulose filters. Filters were immersed in quench solution (A), quench solution supplemented with SF from Patient RA 1 (B), or SF from Patient OA 1 (C) before application of anti-gp78 MAb. Competitive inhibition of SF from RA (B), but not from OA (C) against binding of anti-gp78 MAb to gp78 bound to the filter are shown.

DISCUSSION

AMP has been reported to stimulate the motile activity of self-producing human melanoma cells. Various tumor cells, such as human squamous cell carcinoma, fibrosarcoma, murine melanoma, osteosarcoma, and fibrosarcoma, secrete AMP into culture medium. The cell surface glycoprotein gp78 has been identified as a receptor for AMP(23). The expression of gp78 is closely related to malignant development of tumor cells, especially the invasion and metastatic phenotypes(16). The normal fibroblast, BALB/3T3 clone A31, exhibits motile response to AMP with the expression of gp78(16), suggesting motility regulation.

Figure 3. Dot blot analysis. Three microfilters of SF from Patient RA 1 diluted in PBS (×2 or ×4) were blotted on nitrocellulose filters. Filters were immersed in quench solution overnight, then incubated 3 h in quench solution in the presence (+) or absence (−) of anti-gp78 MAb, indicating few molecules recognized by the anti-gp78 MAb were present in the RA SF.

Figure 4. Effects on cell motility of Gc-4 PF cells of rheumatoid SF treated (b) without or with trypsin digestion for (c) 0, (d) 10, (e) 30, (f) 120 min or (g) with heat inactivation. No SF was added to the control group (a). All data are means ± S.E. The motility factor in rheumatoid SF is a trypsin and heat-labile molecule.

Figure 5. Effects of fractionated rheumatoid SF (Patient RA 1) by DEAE anion exchange column on motile activity of Gc-4 PF cells. Activity in rheumatoid SF (second bar, 50%) and in unbound and bound materials eluted at various concentrations of NaCl (shaded bars) was assessed. All data are means ± S.E. Motile activity was eluted at higher salt concentration, indicating the activity is possibly acidic.

Figure 6. Effects of several blockers of AMP-gp78 signal transduction pathway on motility stimulated by rheumatoid SF (Patient RA 1). Gc-4 PF cells were treated with pertussis toxin (PT) (500 ng/ml), staurosporine (10 μM), genistein (10 μM), or H-8 (10 μM) prior to interaction of the cells with (shaded bars) or without (open bars) rheumatoid SF. All data are means ± S.E. The motility stimulated by rheumatoid SF involves reactions of G-proteins, C-kinases, and tyrosine kinases, but not A kinase.

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by AMP through g78 is not unique to tumor cells. G4-PF cells in this study secreted AMP2, and exhibited high motile and metastatic response to AMP with augmented expression of g78. By using this tumor cell system, AMP-mediated motility that binds to g78 was shown to be expressed in rheumatoid SF2 in the present study. We identified cell motility activity in the SF of patients with OA, and joint trauma. Immunoblot analysis showed that molecules that bind to g78 were absent in the SF of patients with OA or trauma, while rheumatoid SF did contain the motility factors. Furthermore, motile activities expressed in SF were found in RA serum, but not in serum from patients with OA. The expression of motility stimulating activity that may interact with g78 thus appears to be specific for RA, and the molecular species of the motility activity may thus differ from that expressed in patients with OA and joint trauma.

Rheumatoid SF inhibited the binding of anti-gp78 Mab to the antigen in immunoblot analysis, suggesting the presence of an AMP-like factor in SF, as reported. When purified AMP and tumor cell motility activity in rheumatoid SF and AMP. Cell motility activity in rheumatoid SF was heat labile and trypsin sensitive, corresponding to AMP2. When we purified AMP from the culture medium of G4-PF cells in the previous study, AMP was recovered from a DEA/EG exchange column in the unbound fraction and eluted at high salt concentration, 500 mM. In this study, SF from patients with RA was fractionated with the same ion exchange column and buffer, and cell motility activity was eluted, indicating this is consistent with the AMP purification pattern of AMP. We could not determine why activity was not eluted in the unbound fraction, as reported. AMP2 may be responsible for this. The AMP SF14 added to the suspension of RA cells was not converted to AMP, and the concentration was much higher (roughly 500-fold). Presumably, the differences may contribute to the identical expression of homologous molecular aggregate formation, which may alter the net charge in molecules in the low salt buffer2, leading to a single peak elution pattern corresponding closely to the finding that AMP is an acidic protein with pH of 4.8.

The signal transduction pathway and regulatory mechanisms by which AMP affects cells via its receptor have been elucidated in part. Cell motility stimulated by AMP is inhibited by pertussis toxin specifically, suggesting the involvement of guanine nucleotide binding protein (G-protein) in the signal transduction of AMP. The amino acid sequence indicated by g78/AMF cDNA includes a nucleotide binding consensus sequence commonly found in the kinase receptor family2. Kohn, et al reported AMP-stimulated J- tyrosine kinase activation in human melanoma cells. In addition to the classical signaling pathways, Tiruchinappai et al described the involvement of 12(2)-hydroxy-icosatetraenoic acid, a lipoxigenase metabolite of arachidonic acid2.

We observed using specific kinase inhibitors that osteosarcoma cell motility is dependent on protein kinase C and tyrosine kinase reaction, but independent of protein kinase A2.

A cancer cell is often characterized as metastatic in vivo via the adenylate cyclase pathway via kinase A is thought not to be involved in the AMP signaling pathway2. This agrees with the finding that cholera toxin does not inhibit AMP stimulating cell motility. In this study, cell motility activity in rheumatoid SF was inhibited by pertussis toxin, and stau- rorosopine or genistein caused significant decreases in stimulated motility. G-protein mediated and kinase C dependent osteosarcoma cell motility was thus found to be cell motility stimulated by AMP. If AMP inhibition failed to inhibit AMP stimulated motility completely, and if the AMP homologous factors in SF induces osteosarcoma cell motility. But how is AMP homologous factors in SF induce autocrine motility and osteosarcoma cell motility.

In conclusion, this biochemical investigation shows a specific motility factor in rheumatoid SF that might be AMP. So far, we have not been able to clone the gene encoding the origin of AMP. However, numerous lines of evidence suggest that chemokine family members in SF induce osteosarcoma cell motility, because this finding may contribute to the origin of AMP.
